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Resolution of the ATP-dependent proteolytic system from reticulocytes: A component that interacts with ATP

(protein degradation/energy/cell-free system)

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ABSTRACT **The ATP-dependent proteolytic cell-free system from reticulocytes has been resolved into three components, each of which is absolutely required for acid solubilization of ^{125}I-labeled bovine serum albumin radioactivity. In addition to the previously reported heat-stable polypeptide [Ciechanover, A., Hod, Y. & Hershko, A. (1978) *Biochem. Biophys. Res. Commun.* 81, 1100–1105], we now describe a protein of high molecular weight (≈450,000) that is labile at 42°C. The extremely heat-labile factor is remarkably stabilized by ATP. GTP and CTP, which do not stimulate proteolysis, do not stabilize this factor. Adenylate nucleotides such as ADP or the nonhydrolyzable β,γ imido or methylene analogues of ATP cause stabilization although they do not activate proteolysis. A third protein component of the protease system, stable at 42°C, has been separated from the heat-labile species by salt precipitation. All three components are required with ATP for proteolytic activity, but thus far only the heat-labile factor has been shown to interact directly with ATP.**

A universally observed aspect of intracellular protein degradation is the marked dependence on adequate levels of ATP (1, 2) as shown by its almost total depression in the presence of a wide range of inhibitors of energy production. Only recently have there been reports of ATP-dependent proteolysis in cell-free extracts. Goldberg and colleagues have found that ATP stimulates protein degradation in cell-free extracts of reticulocytes (3) and *Escherichia coli* (4), and Roberts *et al.* showed that the proteolytic cleavage of bacteriophage λ repressor *in vitro* requires ATP (5). We have studied the role of ATP in the degradation of abnormal globin chains in intact reticulocytes and in their soluble extracts and have found that in both cases ATP is required at or before the initial cleavage of the complete polypeptide molecule (6).

To gain insight into the mechanisms by which ATP participates in protein breakdown, purification and characterization of the responsible enzymes are required. We have reported (7) that ATP-dependent protease acting on labeled globin can be fractionated into a heat-stable protein that is not retained on DEAE-cellulose (fraction 1) and a crude fraction eluted with 0.5 M KCl. The active principle of fraction 1, now designated APF-1, is a heat-stable and relatively small ($M_r \approx 9000$) polypeptide that has no proteolytic activity but restores ATP-dependent proteolysis when combined with fraction II (7). We report here the further resolution of fraction II into two complementary activities, and identify one of the components as capable of interacting directly with ATP.

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MATERIALS AND METHODS

Fractionation of Reticulocyte Extracts. Soluble lysates were prepared from ATP-depleted rabbit reticulocytes and separated on DEAE-cellulose into fractions I and II as described (7), except that the column was washed with a solution containing 20 mM KCl, 1 mM dithiothreitol, and 3 mM potassium phosphate (pH 7.0) before the elution of fraction II. APF-1 was purified from fraction I by heat treatment, ammonium sulfate precipitation, and gel filtration on a column of Sephadex G-75, as described (7).

Fraction II was separated by ammonium sulfate fractionation to fractions IIA (0–38% saturation) and IIB (42–75% saturation). The precipitates were dissolved in a minimal volume of 20 mM Tris·HCl (pH 7.2)/1 mM dithiothreitol and passed through Sephadex G-25 equilibrated with the same buffer. ATP (0.5 mM) was added to fraction IIA and both fractions were stable when stored at −80°C.

Assay of ATP-Dependent Proteolysis. ^{125}I-Labeled bovine serum albumin (^{125}I-albumin) (2×10^4–10^5 cpm/μg) was prepared as described (8) and passed through a Sephadex G-25 (fine) column (0.9 × 60 cm) to remove acid-soluble material. Unless otherwise stated, the reaction mixture contained in a final volume of 100 μl: 100 mM Tris·HCl (pH 7.6), 2 mM dithiothreitol, 2 mM ATP, 5 mM $MgCl_2$, 1 μg of ^{125}I-albumin, 3.6 μg of purified APF-1, and other enzyme fractions as specified. After incubation at 37°C for 2 hr, the reaction was stopped by chilling in ice, and 0.1 ml of carrier bovine serum albumin (100 mg/ml) was added, followed by 0.8 ml of 5% trichloroacetic acid. After centrifugation, radioactivity in the clear supernatant was determined. Acid-soluble radioactivity in zero-time samples (about 2–3% of the total) was subtracted and the results were expressed as the percentage of ^{125}I-albumin converted to acid-soluble material.

Materials. For protein iodination, carrier-free Na^{125}I was obtained from Amersham and crystalline bovine serum albumin from Sigma. All nucleotides were purchased from Sigma, except for adenosine 5′-[β,γ-imido]triphosphate (Ado*PP*[NH]*P*), which was from Nutritional Biochemicals. Yeast hexokinase (140 units/mg) was obtained from Boehringer, and creatine kinase (115 units/mg) from Sigma.

Abbreviations: APF-1, active principle of fraction 1 [previously designated Fraction I (7)]; APF-2 is defined as the 0–38% saturated ammonium sulfate precipitate from Fraction II (7); Ado*PP*[CH_2]*P*, adenosine 5′-[β,γ-methylene]triphosphate; Ado*PP*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; ^{125}I-albumin, ^{125}I-labeled bovine serum albumin.

Table 1. Separation of fraction II into complementing activities

Fraction added			Degradation of ^{125}I-albumin, %	
IIA, μl	IIB, μl	APF-1	−ATP	+ATP
10	—	+	0.3	0.7
—	10	+	1.3	0.7
2	10	+	—	11.7
10	5	+	—	13.0
10	10	+	0.2	21.1
10	10	−	—	1.4

Fractions IIA (32.4 mg of protein per ml) and IIB (30.0 mg/ml) were added at the amounts specified. Where indicated, the mixtures were supplemented with ATP (2 mM) or purified APF-1 (3.6 μg).

RESULTS

Resolution of Fraction II into Complementing Activities. Initial attempts to separate or purify the active components in fraction II were hindered by its extreme lability and high and variable content of ATP-independent protease activity (7). The lability problem was solved, at least in part, by the finding that ATP stabilizes the labile component (see below), while the high protease background could be minimized by using a suitable substrate that is not hydrolyzed significantly by these enzymes. We found that, in contrast to other polypeptide substrates such as globin (7), ^{125}I-albumin is attacked only slightly by the non-ATP-dependent proteases, whereas it is effectively proteolyzed by the ATP-dependent system. Control experiments showed that: (*i*) ^{125}I-albumin is not cleaved significantly even to large (acid-insoluble) fragments in the absence of ATP, according to autoradiograms of 15% polyacrylamide/0.1% sodium dodecyl sulfate slab gels in which fragments below 20,000 daltons would have been detected; (*ii*) more than 90% of the acid-soluble radioactive material produced in the presence of ATP is iodotyrosine, as analyzed by paper chromatography with 1-butanol/glacial acetic acid/water, 12:3:5 vol/vol (9).

By utilizing the assay system indicated earlier, crude fraction II could be readily resolved into two mutually dependent activities by ammonium sulfate fractionation (see Table 1). Each of fractions IIA (0–38% cut) and IIB (42–75% cut), when incubated without the other, had very little proteolytic activity in either the presence or the absence of ATP, but ATP-dependent proteolysis was reconstituted upon the combination of these two fractions. Activity was proportional with increasing amounts of either fraction IIA or IIB, until it became limited by the complementing fraction. As in the case of crude fraction II, ATP-dependent proteolysis in the reconstituted IIA + IIB system was almost completely dependent upon the presence of APF-1 (Table 1).

Stabilization of the Activity of Fraction IIA by ATP. It was observed previously that fraction II activity is extremely heat labile (7). We have now found that this is due to the lability of fraction IIA, whereas the activity of fraction IIB is relatively stable at 42°C (Fig. 1). The half-time of inactivation of fraction IIA at 42°C varied between 10 and 30 min in different preparations. We furthermore found that ATP effectively protects fraction IIA activity against heat inactivation (Fig. 1).

By utilizing ATP to stabilize fraction IIA activity during gel filtration chromatography, the molecular size of the factor could be determined (Fig. 2): it is large, eluting around the region of ferritin on a Sepharose 4B column (i.e., M_r 450,000). We call this ATP-stabilized high-molecular-weight component in fraction IIA factor 2 of the ATP-dependent proteolytic system (APF-2).

It might be asked whether the apparent stimulation of protein degradation by ATP is merely due to the stabilization of APF-2 activity during assay. It may also be that ATP stabilizes this factor by its phosphorylation. Comparison of the action of various nucleotides on the stimulation of protein breakdown

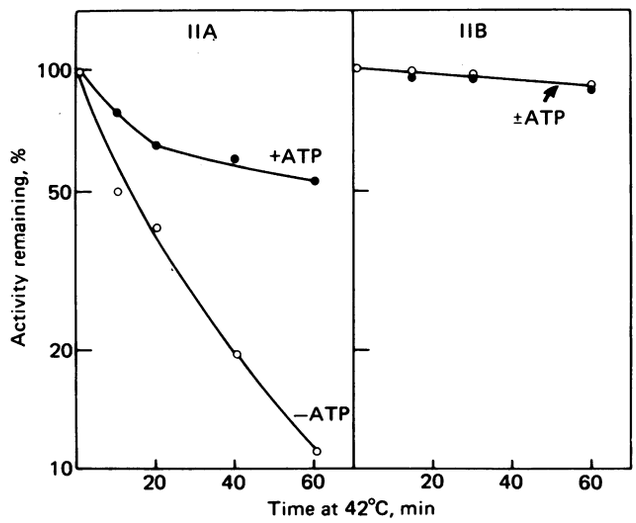

FIG. 1. Heat inactivation of fraction IIA and its protection by ATP. Fraction IIA was diluted to a concentration of 6.5 mg of protein per ml in 20 mM Tris·HCl buffer (pH 7.6) containing 5 mM $MgCl_2$ and 1 mM dithiothreitol and heated at 42°C in the presence (●) or absence (○) of ATP (2 mM). At different times, 10-μl aliquots were withdrawn and assayed with 2 mM ATP, 150 μg of fraction IIB, and 3.6 μg of APF-1. Fraction IIB was diluted and heat treated similarly, and assayed with 165 μg of untreated fraction IIA.

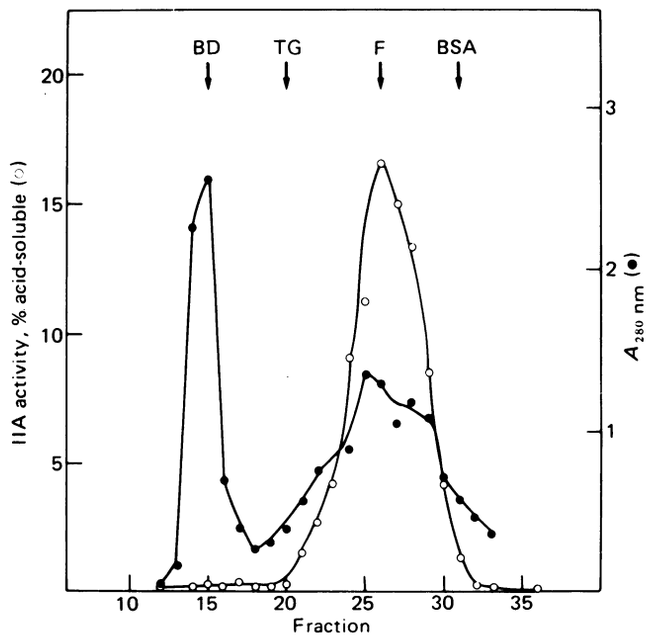

FIG. 2. Gel filtration analysis of fraction IIA on Sepharose 4B. A column of Sepharose 4B (0.9 × 48 cm) was equilibrated with a buffer consisting of 20 mM Tris·HCl (pH 7.6), 1 mM dithiothreitol, and 0.5 mM ATP. Fraction IIA (15 mg) was applied and eluted with the above buffer. Fractions (0.95 ml) were collected and activity was assayed in 50-μl aliquots with fraction IIB (140 μg) and partially purified APF-1 (14.5 μg). Assay conditions were as described under *Materials and Methods*, except that creatine phosphate (10 mM) and creatine kinase (100 μg/ml) were added with ATP (0.5 mM). The absorbance of ATP at 280 nm was subtracted from the total absorbance. Markers (arrows): BD, blue dextran ($M_r > 2 \times 10^6$); TG, thyroglobulin ($M_r \approx 6.7 \times 10^5$); F, ferritin ($M_r \approx 4.6 \times 10^5$); BSA, bovine serum albumin (M_r 69,000).

and the stabilization of APF-2 rules out the above possibilities. Stimulation of protein degradation is specific for ATP. ADP is not effective unless an ATP-generating system such as creatine phosphate and creatine kinase is added; unlike a recently discovered protease from liver cytosol (unpublished data), the reticulocyte system is neither activated nor stabilized by creatine phosphate. Other nucleoside triphosphates, such as GTP, CTP, or UTP, as well as the nonhydrolyzable β,γ imido or methylene analogues of ATP, cannot replace ATP in the activation of proteolysis. In fact, Ado*PP*[NH]*P* inhibits ATP-stimulated protein degradation (not shown). The stabilization of APF-2 is also specific for adenine nucleotides (GTP, CTP, or UTP do not stabilize). However, ADP (but not AMP) and the ATP analogues also partially protect APF-2 against heat inactivation. Furthermore, while Mg^{2+} is absolutely required for ATP-dependent protein degradation, ATP can effectively stabilize APF-2 also in the absence of Mg^{2+} ions (Table 2). It is evident, therefore, that APF-2 is not stabilized by phosphorylation; neither is the stimulation of proteolysis by ATP solely due to the stabilization of APF-2, otherwise the other stabilizing nucleotides would also stimulate protein degradation. It rather seems that the interaction of ATP, its analogues, or ADP with adenine nucleotide site(s) on APF-2 converts this factor to a more stable conformation.

Continued Requirement for ATP in the Action of All Three Components. The question arises as to whether one of the components is an ATP-stimulated protease that produces initial cleavage(s) in the polypeptide substrate, whereas the other factors are ordinary (non-ATP-dependent) proteases or peptidases that convert the cleavage fragments to acid-soluble products. In fact, most of the reticulocyte aminopeptidase and trypsin-like protease activities are present in fraction IIB (unpublished results). To examine this possibility, the reaction mixture was preincubated without one of the components and then ATP was trapped with hexokinase and glucose and the missing factor was added. If one of the components is a terminal protease, it should under these conditions act on the primary cleavage products of ^{125}I-albumin (which should accumulate during preincubation), even after the removal of ATP. In the complete reaction mixture, the addition of hexokinase with glucose immediately arrested ATP-dependent proteolysis, either at the onset of the incubation or later (Fig. 3*A*). Control

Table 2. Nucleotide specificity of the stabilization of APF-2

Addition	Relative activity: Stimulation of protein breakdown	Relative activity: Stabilization of APF-2
ATP	1.00	1.00
ATP, $-Mg^{2+}$	0	0.98
ADP	0	0.56
ADP + creatine-*P* + kinase	1.57	—
AMP	0	0
Ado*PP*[NH]*P*	0	0.53
Ado*PP*[CH_2]*P*	0	0.54
GTP	0	0
CTP	0	0
Creatine-*P*	0	0

All nucleotides were added to give 2 mM, creatine phosphate to 15 mM, and creatine kinase to 100 μg/ml. Stimulation of protein breakdown is expressed relative to the activity obtained with ATP, which was 17.4% of ^{125}I-albumin solubilized. Stabilization of APF-2 was tested by treating at 42°C for 25 min. % ^{125}I-albumin degraded was: untreated, 14.3; heated without additions, 2.5; heated with ATP, 10.0. In the case of Ado*PP*[NH]*P*, the results were corrected for the inhibition (38%) caused by a similar final concentration of the analogue in the assay mixture.

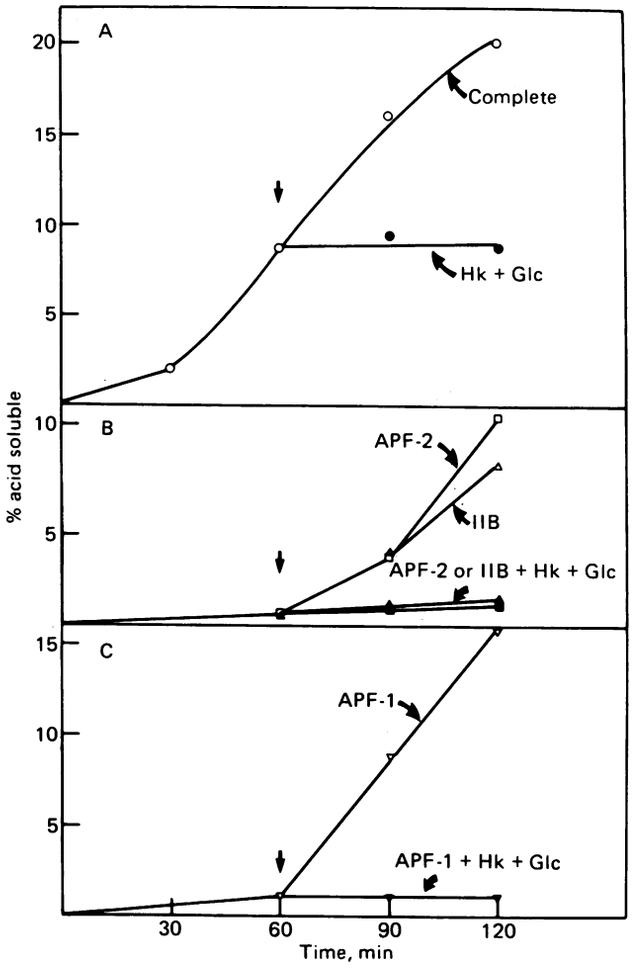

FIG. 3. Continued requirement for ATP for the action of the various components. The complete system contained (μg of protein): APF-1, 3.6; APF-2, 81; fraction IIB, 150. At 60 min, where indicated, hexokinase (Hk, 1 μg) and glucose (Glc, 10 mM) were added. (*A*) Complete system. (*B*) Preincubated at 37°C for 60 min without either APF-2 or fraction IIB. (*C*) Preincubated without APF-1. In preincubation, all components of the reaction mixture (except for the one omitted) were incubated with ^{125}I-albumin and ATP, and then the missing component was added without (open symbols) or with (closed symbols) hexokinase and glucose.

experiments showed that hexokinase or glucose, each without the other, did not significantly inhibit protein degradation. When the reaction mixture was preincubated without APF-2, fraction IIB (Fig. 3*B*) or APF-1 (Fig. 3*C*) and the missing factor then added, the restoration of protein degradation was completely inhibited by the ATP trap in all cases. This shows that the action of all three components requires the continued presence of ATP, and no single component is an ATP-independent terminal protease or peptidase.

Examination of the time course of protein degradation in the above experiments shows that, in the complete reaction mixture, there is a time lag in the degradation of ^{125}I-albumin during the initial 30 min of incubation, following which the reaction proceeds linearly (Fig. 3*A*). Preincubation without APF-2 or fraction IIB (followed by their respective addition) did not abolish the lag significantly (Fig. 3*B*). However, when the mixture was preincubated without APF-1 (i.e., ^{125}I-albumin incubated in the presence of APF-2, fraction IIB, and ATP), and then APF-1 was added, the reaction immediately proceeded at a high linear rate (Fig. 3*C*). These observations may indicate that the rate of formation of the active ATP-dependent

protease complex may be limited by interaction of APF-2 and fraction IIB (see *Discussion*).

DISCUSSION

ATP-dependent protein degradation in reticulocytes is apparently carried out by a complex system consisting of at least three components of widely differing properties. Among the three components that have been resolved, the high-molecular-weight factor described in this paper (APF-2) has been identified as interacting directly with ATP by virtue of the stabilization at 42°C resulting from ATP. Phosphorylation of APF-2 cannot be part of the mechanism of stabilization because of the protective action of nonphosphorylating adenine nucleotides as well (Table 2). However, APF-2 might be a protein kinase in the proteolysis reaction: the stabilization of an erythrocyte protein kinase by ATP has been described (10), and preparations of APF-2 contain considerable protein kinase activity with endogenous proteins as acceptors (unpublished observations). However, APF-2 cannot be identical to the above erythrocyte enzyme, because APF-2 activity is lost during reticulocyte maturation (unpublished observations), as is protein degradation in intact cells (6).

The elucidation of the mode of action of APF-2, or the role of ATP, depends upon further resolution, purification and characterization of the various components. ATP cannot act simply by stabilization because nonphosphorylating ATP analogues cannot replace ATP in the protease assay. ATP may act by the modification (such as phosphorylation or adenylylation) of an enzyme or a protein factor or by the modification of the polypeptide substrate, or it may be required for the interaction of these macromolecules. If a phosphorylation reaction occurs, it will have to be concluded that dephosphorylation is also very rapid in the partially purified system, because the addition of an ATP trap stops protein breakdown immediately (Fig. 3 *B* and *C*). In the case of a phosphorylation mechanism, this might mean that the latter factors are either continuously phosphorylated (and dephosphorylated) themselves, or are closely related to the phosphorylation process.

The observed lag in the time course of the reaction and its abolishment by the preincubation of all components except APF-1 might mean that this heat-stable polypeptide enters a functional complex rapidly at a step subsequent to the initial interaction of APF-2 and fraction IIB. However, other explanations cannot be eliminated at this stage of purification. For example, fractions APF-2 and IIB may contain denatured proteins that serve as substrates and compete with ^{125}I-albumin; these denatured proteins might be rapidly broken down during preincubation by other non-ATP-dependent proteases present in these fractions.

The multi-component reticulocyte system appears to differ from the ATP-dependent proteolytic cleavage of the phage λ repressor (5). In the latter, the reaction apparently requires only one protein, recently identified as the product of the *E. coli recA* gene (11). Though the *recA* protein carries out a limited endoproteolytic cut in a specific polypeptide, while the reticulocyte system breaks down various proteins to free amino acids, some similarities in the mechanisms of the involvement of ATP in peptide bond cleavage might be expected. It should be noted that the possible contamination of purified *recA* protein by slight amounts of other factors(s) of high activity was not ruled out (11). It is also possible that the *recA* protein is a multifunctional enzyme in which different sites of the enzyme molecule catalyze different functions. These different functions may be analogous to some of those carried out by the separate components of the reticulocyte system.

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